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Stem Cells in Pancreatic Cancer

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1. Introduction

Pancreatic cancer is the fourth most frequent cause of cancer-related deaths; it also represents one of the most aggressive cancer types, with a high incidence of distant metastasis and mortality [1]. The detection of pancreatic cancer at early stages, the prediction of the potential resectability, or the response to therapy are the current major challenges in improving the clinical outcome of pancreatic ductal adenocarcinoma (PDAC) [2]. The main issue against successful therapy is represented by the absence of early diagnostic and prognostic markers, as well as the unresponsiveness to radiation and chemotherapies [3]. Among other factors that contribute to the lack of success in the therapy of pancreatic malignancies, cancer stem cells (CSCs) appear to have a major role. Cancer is characterized by cellular heterogeneity; CSCs, which represent a distinct subpopulation of cells, seem to be responsible for tumor initiation and persistency, due to their properties of self-renewal and multilineage differentiation. CSCs are considered as best candidates responsible for tumorigenesis, metastasis, and chemo- and radio-resistance [4]. Understanding and properly addressing the challenge represented by CSCs appears as a logical, yet difficult task in anti-cancer strategies.

2. Cancer stem cells: Involvement in the progression, invasion and metastasis

2.1. Pancreatic cancer stem cells (CSCs) phenotyping and isolation

Cancer stem cells from epithelial tissues were identified for the first time in breast cancer in 2003, when Al-Hajj et al. reported that a distinct population of cells, CD44⁺CD24⁻/low

epithelial-specific antigen (ESA+), develops tumors in immunodeficient mice [5]. In pancreatic cancer, the presence of CSCs was reported in 2007 by Li C *et al*, who showed that CD44⁺CD24⁺ESA⁺ cells possess highly tumorigenic potential [6].

Similar to other types of cancer, pancreatic tumor cells apparently grow around a population of CSCs which are capable of promoting tumor growth and progression through many mechanisms, including alteration of adjacent stromal cells and evasion of conventional therapies [7]. Therefore, their identification, isolation and further *in vitro* studies represent the field that provided the most important breakthroughs in pancreatic cancer. The phenotypic characterization of CSCs is an ongoing process, however, there are some biomarkers that are recognized as significant for the stemness phenotype: CD133, Nestin, Notch1-4, Jagged 1 and 2, ABCG2 and aldehyde dehydrogenase (ALDH1) [8]. Following the model of breast cancer stem cells [5], a pancreatic CSC subpopulation was shown to be epithelial-specific antigen (ESA)⁺/CD44⁺, but unlike the first, also CD24⁺ [6]. CD44⁺CD24⁺ESA⁺ cells represent 0.5% to 1.0% of all pancreatic cancer cells [4] and show self-renewal capacity *in vitro*, are capable of forming tumor spheres, and can be passaged multiple times without loss of tumor sphere-forming capability [9, 10].

CD133 is a biomarker for putative CSC in several solid tumors [11] and it was used as a marker for flow cytometry to select a subpopulation of tumor cells able to generate tumors in athymic mice [12]; it has been reconfirmed in later studies, by immunohistochemistry, to be present in ductal adenocarcinomas [13]. Furthermore, double positive CD133⁺/CXCR4⁺ seem to be preferentially located in the migration front of pancreatic tumors [12] and demonstrate increased metastatic abilities [14].

Along with CD133, aldehyde dehydrogenase 1 (ALDH1) is also considered a useful marker of stemness, both of which are currently being used for flow cytometry sorting of stem-enriched side populations [15]. Increased activity of ALDH1 was associated with CSCs and has been correlated with invasion, migration and poor overall survival in patients with pancreatic cancer [16]. Therefore, ALDH (+) cells have stem and mesenchymal cell features and are more tumorigenic than CD44⁺/CD24⁺ cells [17]. An intriguing and somewhat discouraging observation is that only 0.015% of all tumor cells are concomitantly ALDH⁺ and CD44⁺/CD24⁺, yet ALDH⁺ cells alone have potent tumorigenic activity, thus, several subsets of tumor-initiating cells might be present within a pancreatic tumor [18].

The majority of CSCs is not positive for cytokeratins (intermediate filament proteins present in differentiated epithelial cells) [12], but for Nestin – an intermediate filament protein and a stem cell marker associated with cell integrity, migration, and differentiation. In pancreatic carcinoma, one third of tumor cells present nestin expression which is correlated with tumor staging and metastasis. Nestin-expressing cells are involved in epithelial-to-mesenchymal transition (EMT) and seem to be the origin of pancreatic intraepithelial neoplasia lesions [19]. Recently, presence of Nestin in various types of malignancy was associated with tumoral angiogenesis and was proposed as an angiogenic marker [20].

Within a recent study, authors comparatively analyzed cancer stem cell markers in normal pancreas and pancreatic ductal adenocarcinoma, yielding surprising results: although

expression was increased, neither CD133, nor Notch proteins or ALDH1 reached statistical significance; in turn, Jagged 1 was shown to be a robust marker, along with Nestin [8].

Mouse models of ductal pancreatic neoplasia seem to harbor a subpopulation of cells expressing high levels of doublecortin-like kinase 1(DCLK1), alpha tubulin acetyltransferase 1(ATAT1), hairy and enhancer of split-1(HES1), hairy/enhancer-of-split related with YRPW motif 1(HEY1), Insulin-like growth factor 1 receptor (IGF1R), and Abelson murine leukemia viral oncogene homolog 1 (ABL1) with cancer-initiating properties. As this subpopulation is identifiable at very early stages during adenocarcinoma development, it provides new targets for early diagnostic and drug testing [21].

All the studies suggest the importance of CSCs in the prognostic and therapeutic responses of pancreatic cancer patients and underline the necessity of stem cell surface marker characterization. In this regard, it is useful to better understand the basic genetic and epigenetic processes of cancer stem cell transformation from highly regulated stem cells and also the interaction between stem cells and the tumor niche [22].

2.2. Epithelial-to-mesenchymal transition

Recent studies suggest the involvement of CSCs in the progression, aggressiveness and epithelial-to-mesenchymal transition (EMT) in pancreatic cancer [23, 24].

The epithelial-to-mesenchymal transition concept was first described 40 years ago, in relation to the development of the embryo and germ layer formation [25]. Since then, EMT has been shown to be a key player in several normal biological processes or pathologies, such as: embryogenesis, wound healing or cancer progression. The process is essentially defined by phenotypic changes of epithelial cells towards mesenchymal cells. During embryogenesis, EMT represents the biological process in which cells from the epithelial compartment detach, migrate and acquire a mesenchymal phenotype required for the formation of the mesoderm [26]. EMT also plays a key role upon wounding; the wound healing process is marked by epithelial cell migration to the site following EMT signals from the surrounding tissues and acquisition of the mesenchymal-like phenotype [27]. During this process, changes occur in the expression of specific genes, epithelial cell down-regulation of adherent and tight junction proteins (Claudin1 and 7, Occludin and E-cadherin) and matrix metalloproteinase-increased activity, resulting in increased mobility [28]. The major embryonic signaling pathways Wnt, Notch, Hedgehog and Transforming growth factor beta (TGF- β) are involved in upregulation of EMT-activating transcription factors, including Snail, Twist and Slug families [29]. TGF- β signaling, associated with other signaling pathways like Ras/MAPK, is essential for EMT process by repressing junction components like E-cadherin, Claudins, and Occludin via Snail transcription factors. TGF- β is also involved in carcinogenesis, playing dual roles by acting as a tumor suppressor in early tumor development, and paradoxically, by promoting tumor cell invasion in later stages [30].

Wnt signaling is also involved in the EMT program, by stabilizing Snail and β -catenin levels and by blocking Glycogen synthase kinase 3 (GSK-3 β) activity, processes also related to

cancer metastasis. On the other hand, Snail can interact with β -catenin and it enhances Wnt signaling [31].

Notch signaling is responsible for cell fate, proliferation, differentiation, apoptosis and the maintenance of stem cells and also for hypoxia, which can activate EMT in cancer [32]. It is also considered that Notch can regulate endothelial and mesenchymal markers to sustain mesenchymal transformation [33]. Notch pathways have been shown to increase cellular migration by activating Nuclear factor kappa β (NF- κ B), Matrix metalloproteinase 9 and Vascular endothelial growth factor (VEGF) in pancreatic cancer cells [34]. More studies suggest that Notch inhibition can reverse EMT in the Mesenchymal-to-Epithelial Transition (MET) and can be considered a promising therapeutic strategy in cancer treatment [35].

Hedgehog signaling is also involved in embryonic cell growth and organogenesis as well as in regulating genes associated with cell proliferation, differentiation, and cell motility [36]. Some studies showed that the Hedgehog pathway, normally quiescent in adult organs, is very active in cancer where it can increase stromal hyperplasia, myofibroblast differentiation, and production of extracellular matrix, enabling the EMT process in cancer cells [37].

A solid body of literature shows that the EMT process is actively implicated in tumor metastasis and tumor recurrence and that cancer stem cells that have undergone EMT display resistance to therapy [38, 39]. The accepted theory is that CSCs from solid tumors acquire migratory potential together with mesenchymal transition, migrate from the primary tumor, colonize other tissues and form a new metastatic tumor with similar characteristics as the initial one (Figure 1) [40, 41]. *In vitro* and *in vivo* studies support EMT involvement in early steps of carcinogenesis, by identifying EMT-associated markers such as mesenchymal-specific markers (i.e. Vimentin and Fibronectin), epithelial specific markers (i.e. E-cadherin and Cytokeratin), and transcription factors (i.e. Snail and Slug) in tumor samples [42]. Moreover, the expression of EMT-specific genes has been identified at the level of the invasive front of primary tumors [32] and reversely, the expression of CSCs markers can be induced by overexpressing Snail or Twist, the most important transcription factors involved in the EMT process [43]. From the other point of view, cancer cells from metastasis after the EMT process can show a CSC phenotype and TGF- β signaling is considered to be a crucial factor involved in these processes [44].

Cellular migratory potential is also increased by up-regulation of Mucin-4 (MUC4) and fibroblast growth factor receptor 1 (FGFR-1) stabilization [45]. Other studies show that the process in pancreatic cancer can also be regulated by Forkhead box protein M1 (FoxM1)-caveolin [46], GLI-Kruppel family member GLI1 (GLI1) [47], hepatocyte growth factor (HGF) or platelet-derived growth factor (PDGF) [48]. Taken into account these observations, EMT-type pancreatic tumor cells represent a highly important research focus for the therapies aiming at reducing or preventing invasion, metastasis and therapeutic resistance in pancreatic cancer.

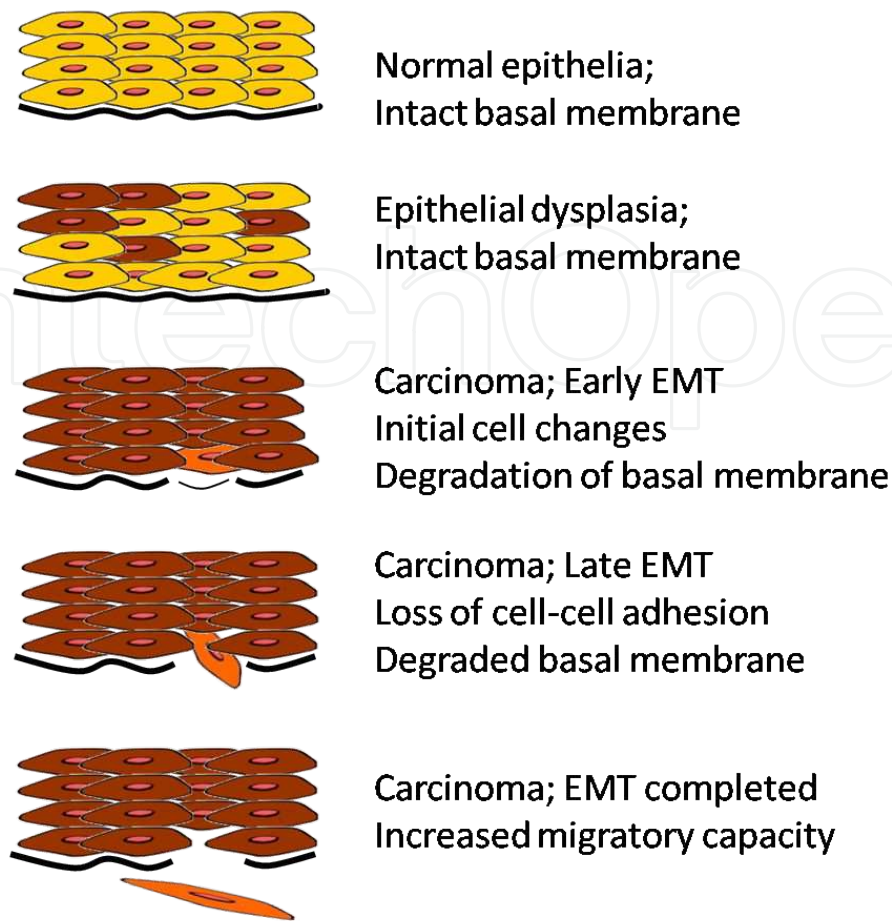


Figure 1. Epithelial-to-mesenchymal transition process

2.3. Regulatory pathways in pancreatic cancer stem cells

Analysis of expression of CSC-related genes in a purified subpopulation of putative pancreatic CSCs showed that up to 46 canonical pathways are upregulated, including human embryonic stem cell pluripotency, tight junction signaling, NF- κ B signaling, Wnt/ β -catenin signaling, integrin signaling, and Ephrin signaling networks [49].

In particular, out of most signaling pathways involved in maintaining self-renewal in normal stem cells, pancreatic CSCs are characterized by overexpression of Sonic Hedgehog (Shh), Wnt, Notch, AKT, NF- κ B, and BMI1 Polycomb Ring Finger Oncogene(BMI-1). Further, signaling pathways which are not dysregulated in metastatic tumors are overexpressed in the pancreatic CSCs [4, 50].

Hedgehog, Notch, Wnt (Figure 2) are shown to be of particular importance in pancreatic cancer stem cells, due to their role in pancreatic embryonic development and differentiation [51]. These signaling pathways are altered in CSCs and EMT-like cells in pancreatic cancer, being involved in self-renewal of CSCs, tumor growth, invasion, metastasis, and resistance to therapy [52].

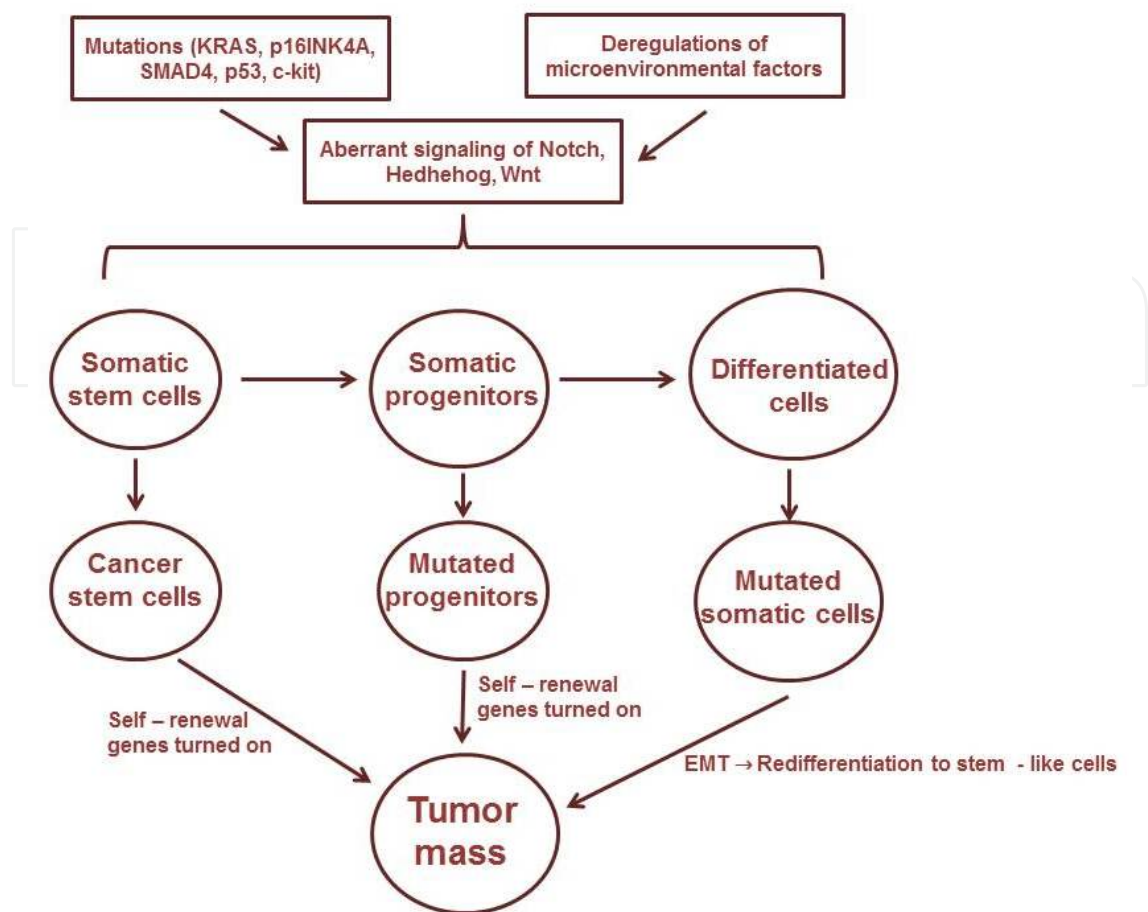


Figure 2. Factors involved in occurrence of cancer stem cells. The emergence of mutations and aberrant signaling in normal stem cells, progenitors, or differentiated cells triggers the transformation of normal cells into cancer stem cells, losing control of cell division.

Notch signaling is involved in the early developmental stages of pancreatic cancer by maintaining epithelial cells in a progenitor state. Tumor cells present an overexpression of Notch signaling, high levels of Notch-1 and Notch-2 while normal pancreas shows a weak expression of pathway-related molecules [53, 54]. Notch signaling is involved in cell proliferation, survival, apoptosis and differentiation of pancreatic cells and can promote EMT by controlling some transcription factors and growth factors like Snail, Slug, and TGF- β . Among Notch target genes are found Akt, cyclin D1, c-myc, cyclooxygenase-2 (COX-2), extracellular signal-regulated kinase (ERK), matrix metalloproteinase-9 (MMP-9), mammalian target of rapamycin (mTOR), NF- κ B, VEGF, p21cip1, p27kip1, and p53, all involved in development and progression of human cancer. Gemcitabine-resistant pancreatic cancer cells present overexpression of Notch-2 and Jagged-1, while Notch1, a key downstream mediator of Kirsten rat sarcoma viral oncogene homolog(KRAS), is responsible for pancreatosphere formation [7, 51, 53]. Overexpression of Notch ligand Delta like ligand 4 (Dll-4) in pancreatic cancer cells promotes expression of octamer-binding transcription factor 4(Oct4) and Homeobox Transcription Factor Nanog(Nanog) (transcription factors essential for both early embryonic development and pluripotency maintenance in ES cells) and thus increases the number of CSCs [55, 56].

Many studies found that pancreatic cancer stem cell resistance to chemotherapy is linked to activated Notch signaling, but the exact mechanism remains unclear [57, 58]. There is more evidence showing that the Notch signaling pathway is essential in supporting KRAS ability to transform normal cells into tumor stem cells. Notch-1 inhibition with specific siRNA or treatment with γ -secretase inhibitors increases apoptosis and decreases proliferative rates, cell migration and invasive properties of pancreatic cancer cells [53]. In this regard, in pancreatic cancer treatment, Notch signaling inhibition can be quite attractive, as long as there is no data arguing that Notch signaling has a critical role in normal adult pancreatic homeostasis [59]. Targeting Notch signaling as a treatment for metastatic pancreatic cancer could prevent the acquisition of the EMT phenotype and resistance to therapy [60].

Hedgehog signaling is another self-renewal pathway, allowing normal stem cells to become independent of control signals; as a result of mutations in this signaling, transformed cells can use Hedgehog for tumor initiation, progression, and metastasis. *In vivo* studies showed that compared to normal pancreatic epithelial cells, CD44+CD24+ESA+pancreatic cancer stem cells present with an up-regulation of Sonic Hedgehog (Shh) transcripts (a ligand of Hedgehog signaling) [61]. Moreover, 70% of pancreatic cancer tissue presents overexpression of Shh, suggesting that Hedgehog signaling may be involved in pancreatic carcinogenesis [51]. Many studies showed that Shh signaling can activate pancreatic stellate cells, promotes fibroblast infiltration, and increases secretion of fibronectin, collagen type I, MMPs, and TGF- β [62]. Studies in the pancreatic cancer cell line PANC-1 showed that inhibition of Hedgehog signaling by Smoothened (Smo) suppression can reverse EMT, induce apoptosis via PI3K/AKT inhibition, and inhibit the invasion of pancreatic cancer cells [63]. Moreover, combination of focal irradiation with Hedgehog signaling inhibition reduces lymph node metastasis in an orthotopic animal model [64].

Wnt/ β -catenin signaling is involved in cell proliferation, migration, apoptosis, differentiation, and stem cell self-renewal in several types of cancer [65]. Wnt/ β -catenin signaling pathway dysregulation is also associated with chemoresistance in pancreatic cancer and recent studies suggest that nuclear β -catenin is essential for the EMT [66, 67]. *In vitro* and *in vivo* studies suggest that activated β -catenin may decrease differentiation of epidermal stem cells, increase self-renewal capacity, and develop epithelial cancers in transgenic mice [68]. Kong D et al. showed that there are some connections between Wnt signaling and Snail, a major regulator of the EMT process. Thus, overexpression of Snail could increase expression of Wnt target genes by interaction with β -catenin [69].

In 2013, Sun L et al. showed that one of the most active signaling pathways in pancreatic cancer stem cells is NF- κ B, whose inhibition leads to loss of stem cell properties. This study also showed that aberrant epigenetic processes, like CpG promoter methylation, can be involved in carcinogenesis mediated by cancer stem cells [70]. These results were confirmed by studies conducted on PANC1 and HPAC pancreatic cancer cell lines [51]. Activity of the pro-inflammatory NF- κ B induces expression of Shh by pancreatic cancer cells and stromal cells, leading to activation of the Hedgehog pathway [71].

Another possible marker for pancreatic CSCs is Met Proto-Oncogene (c-Met), whose inhibition has been correlated with a decrease of tumor growth and with preventing the development of

metastases [1, 72]. c-Met is a receptor tyrosine kinases involved in cell survival, growth, angiogenesis and metastasis. c-Met activates many signaling pathways, including Ras-MAPK, PI3K/Akt NF- κ B, and Wnt/GSK-3 β / β -Catenin and is overexpressed in pancreatic cancer [73].

2.4. MicroRNAs in pancreatic adenocarcinoma

MicroRNAs (miRNAs) are potent regulators of cell function via their role as translational regulators for the synthesis of key proteins. Most often, several miRNAs display different expression profiles in cancer cells, including pancreatic cancers.

MiR-21, miR-155 and miR-17-5p appear upregulated in tumoral cells, and these miRs are often called oncogenic miRNAs [60, 74]. Similarly, a series of miRNAs, referred to as tumor suppressor miRs (miR-34, miR-15a, miR-16-1 and let-7) are downregulated in cancers [54, 75]. Key cell differentiation programs during development are controlled by the members of let-7 (Let-7) and miR-200 families. In cancer, loss of Let-7 leads to disease progression and de-differentiation. The EMT process is also regulated by miRNA-dependent mechanisms and the same Let-7 family appears as a regulator of EMT and of stem cell maintenance. According to Hasselman et al [75], inhibition of maturation of Let-7 by nuclear receptor for the cytotoxic ligand TNFSF10/TRAIL (TRAILR2) in pancreatic cancer cell lines, increases their proliferation. This is consistent with high levels of nuclear TRAIL2 in tissue samples from poor outcome patients.

Pancreatic neoplasms seem also to exhibit their own pattern of miR overexpression, when compared to normal pancreatic tissue: upregulation of miR-93, miR-95, miR-135b, miR-181c, miR-181d, miR-182, miR-183, miR-190, miR-196b and miR-203, miR-767 and miR-1269 and downregulation of miR-20a and miR-29c [76]. In human pancreatic cancer, DCLK1 regulates EMT by a mechanism dependent on miR-200a [77].

MiRNAs were recently considered to have a role in regulation of CSCs [51]. The population of BxPC-3-LN cells (lymph node metastatic pancreatic cells) contains a 5-fold increased population of CD133+/CXCR4+ cells (stem-like cells) compared with the parental (non-metastatic) BxPC-3 cells. Remarkably, a different miRNA pattern is displayed in CSC-like compared with the regular cells: up-regulated miR-572, miR-206, miR-449a, miR-489 and miR-184 were found, as well as downregulated let-7g-3p, let-7i-3p, let-7a-3p, miR-107, miR-128 and miR-141-5p [14].

The miR-200 family members are identified as key regulators of cell maintenance and EMT. It is considered possible that tumor progression is a process resulting in progressive de-differentiation towards a cell type having a stem cell-like phenotype. This process appears to be regulated by miRNA-dependent mechanisms. DCLK1 (a putative marker for pancreatic and intestinal cancer stem cells) regulates EMT in human pancreatic cancer cells via a miR-200a-dependent mechanism [77]; it also acts as a regulator of Let-7a in pancreatic and colorectal cancer cells, supporting the concept that these miRNAs may be novel and relevant targets in solid tumor cancers [78]. Sureban et al demonstrated that DCLK1 inhibition results in up-regulation of miRNAs that negatively regulate some key angiogenic and pluripotency

factors [79]. In AsPC1 tumor xenografts, downregulation of c-MYC and KRAS via let-7a was observed by a similar mechanism demonstrated in pancreatic cancer cells.

Repression of two tumor-suppressor miRs, miR-143 and miR-145, is reported in pancreatic cancer, as well as in other cancers [80]; moreover, experimental restoration of miR 143/145 levels using nano-vector delivery was demonstrated to inhibit pancreatic cancer cell growth [81]. The miR-143/145 cluster cooperates and inhibits the expression of KRAS2 and ras responsive element binding protein 1 (RREB1), its downstream effector [80]. MiR-145 was demonstrated to inhibit cell proliferation in lung adenocarcinoma, by targeting epidermal growth factor receptor (EGFR). In many cancers, including pancreatic cancer, EGFR is upregulated [82], while inhibition of EGF signaling inhibits cancer initiation and progression [83]. Also a suppressive effect of EGFR on miR-143 and miR-145 was demonstrated on models of colon cancer [84]. These findings are indicators of a negative feedback loop between EGFR and miR-143/145, which is similar to KRAS/RREB1 – miR-143/145.

The major role of vascular endothelial growth factor (VEGF) signalling via its receptors, VEGFR1 and VEGFR2, was demonstrated in tumor vascular growth, angiogenesis, and metastasis, while upregulated angiogenic factors in various cancers-colorectal, breast, renal, liver, and ovarian-have been correlated with poor prognosis. Pancreatic ductal adenocarcinoma (PDAC) exhibits endothelial cell proliferation, a mechanisms that increases angiogenesis. Inhibition of VEGF-A, VEGFR1 and VEGFR2 resulted in inhibition of tumor growth and angiogenesis in mouse models of PDAC. Studies and computational analysis outlined a putative binding site for miR-200 (miR-200a, b and c) in the 3' UTR of VEGFR1 and VEGFR2 [85].

Identification of dysregulated expression of various miRNAs, the existence of regulatory loops between miRNAs and protein regulators of key processes (such as cell growth, angiogenesis, differentiation) suggested the need and potential effectiveness of strategies aiming to restore the "normal phenotype" expression pattern of miRNAs for cancer treatment. Various approaches are developed and investigated, such as the delivery of tumor suppressor miRNAs [86], suppression of expression or action of oncomirs [87], targeting the expression of key regulators (such as DCLK1, adenosine monophosphate activated kinase $\alpha 1$ (AMPK $\alpha 1$) [88], leading to miRNAs modulation or even to simultaneous modulation of multiple miRNAs, suggesting that using miRNAs as therapeutic agents or addressing miRNAs as targets represents a potential solution for the therapy of critical cancers.

2.5. CSCs and tumor environment

Although the presence of stromal tissue is described and accepted as a fact in all types of solid cancers, pancreatic adenocarcinoma displays a particularly dense atmosphere of connective tissue, known as "desmoplastic reaction". Since the new cancer paradigm of "stroma-cancer interaction", more thorough investigations have focused on the pancreatic tumor environment, and it is now accepted that the dense connective tissue surrounding malignant cells is at least partially responsible for hindering drug delivery. The pancreatic cancer stroma is now the focus of a new therapeutic approach called "stroma depletion", which can be achieved

through Hedgehog inhibitors [89]. What stromal cells are responsible for Hedgehog signaling responsiveness is currently under investigation, as it would designate them as new anti-cancer targets. Stromal cells are also of importance when considering the concept of stem cell niche – a unique microenvironment involved in generating hierarchies to maintain self-renewal and to control cell fate. The relationship between CSCs and a putative malignant niche is less well stated than for normal stem cells. CSCs are capable of migrating from the original tumor to distance, behavior that is not common for adult, normal stem cells, but is well documented for the hematopoietic stem cell. Stroma of hematopoietic tissue is a particular one, based on reticular connective tissue, unlike most malignant stromas, rich in dense irregular connective tissue. This would possibly indicate the partial independence of CSC from stem-cell niche [90].

a. Pancreatic stellate cells

There is a proven interaction between the CSCs and the tumor stroma, at least in part responsible for increased metastatic abilities of cancer cells. Tumor-stroma interaction is the new cancer paradigm and in the particular case of pancreatic cancer is supported by the presence of pancreatic stellate cells (PSCs) – a subpopulation of desmin-positive periacinar cells, found as well, but in inactive state, in the normal pancreas [91]. Studied at first in relationship with pancreatic fibrosis [92], they were more recently increasingly investigated in the progression of pancreatic cancer [93–95]. In the activated form, stellate cells secrete an array of pro-inflammatory cytokines and promote an immunosuppressive microenvironment [96], secrete various growth factors (e.g. platelet-derived growth factor, stromal-derived factor 1, epidermal growth factor, insulin-like growth factor 1, fibroblast growth factor) [97], as well as matrix adhesion molecules (collagen type I, secreted protein acidic and rich in cysteine (SPARC), small leucine-rich proteoglycans, periostin) and matrix metalloproteinases (MMP-2 and MMP-9), that have been associated with the invasive phenotype of pancreatic cancer cell lines [41]. This particular pattern of pancreatic cell secretome mediates effects on tumor growth, invasion, metastasis and resistance to chemotherapy and is modulated by CSCs, through release of mitogenic and fibrogenic stimulants, such as Transforming Growth Factor β 1 platelet-derived growth factor, sonic hedgehog, galectin 3, endothelin 1 and serine protease inhibitor nexin 2 [97]. Recognition of their importance in tumoral behaviour led efforts to isolate, cultivate and immortalize them for further manipulation with therapeutic purposes [98–100]. Upon activation, pancreatic stellate cells suffer a shift of phenotype towards myofibroblast morphology and a subsequent switch of protein expression [101]. Indirect co-culture of pancreatic cancer cells with PSCs seem to favor the stem phenotype of cancer cells, as evaluated by Hamada et al. by the spheroid-forming ability of cancer cells and expression of cancer stem cell-related genes ABCG2, Nestin and LIN28. In addition, co-injection of PSCs enhanced tumorigenicity of pancreatic cancer cells *in vivo* [90]. The presence of α smooth muscle actin (α SMA) in activated pancreatic stellate cells leads to association with cancer-associated fibroblasts (CAFs) – a cancer modified subpopulation of fibroblasts, identified by the very same marker, that was shown to sustain tumor cells metabolism and favor tumor progression [102]. CAFs also mediate EMT of tumor cells, possibly through a pro-inflammatory signature [103] – secretome that has also been reported in pancreatic stellate cells, not only in cancer but also in chronic pancreatitis [104].

From tumor-stroma interactions new lessons were learned in diagnostics and therapeutics of pancreatic cancer. Secreted Protein, Acidic, Cysteine-Rich (SPARC) (a member of the family of matricellular glycoproteins that is highly expressed in PSCs and the tumour/stroma interface) is now proposed as marker for accurate diagnostic, as 80% of pancreatic ductal adenocarcinomas seem to express it [105]. Due to its ability to bind to basement membrane collagen IV and fibrillar collagens I, III, V and also to bind albumin [106], it has been used to increase distribution of the chemotherapeutic agent paclitaxel within the tumoral mass [107].

Changes within the stem niche, such as hypoxia, are "tuning" the behavior of stem cells, inducing the activation of survival, proliferation, differentiation and angiogenesis.

b. Mesenchymal stem cells – dual facets in cancer

Mesenchymal stem cells (MSCs) are pluripotent cells with homing abilities that are involved in tissue repair, including outside their native niche, that reside primarily in the bone marrow, but also exist in other sites such as adipose tissue, peripheral blood, cord blood, liver, and fetal tissues [108]. They also exhibit a natural tendency of homing into tumors – ability that is starting to be exploited in anticancer treatment, using these versatile cells as cargo delivery for cytotoxic drugs or gene therapy [109]. This behavior has been also reported in pancreatic cancer, by the use of genetically engineered labeled MSCs that efficiently accumulate within the pancreatic tumor, when injected into tumor-bearing mice [110].

Pro-tumor effect of MSCs

Very recent reports have demonstrated that mesenchymal stem cells (MSCs) can function as precursors for CAFs [111, 112]. Interestingly, not all types of MSCs have this particular ability, a recent report from Subramanian et al. arguing that this is not a feature of umbilical-cord derived pluripotent cells [113]. In pancreatic cancer, like in any other type of cancer, these myofibroblast-like cells contribute to inducing EMT in side population cells, maintain tumor-initiating stem cell-like characteristics, including augmenting expression levels of various stemness-associated genes, enhancing sphere-forming activity, promoting tumor formation in a mouse xenograft model, and showing resistance to anticancer drugs [114].

Bone marrow derived progenitor cells were found to participate to neovascularization of tumors [115], a process that was shown to be dependent on Hedgehog signaling [116]. The recruitment of these progenitors is accomplished by CAFs through stroma-cell derived factor 1 (SDF-1) signaling [117].

Anti-tumor activity

An increasing number of reports show that MSCs have the ability of negatively influencing tumor behaviour, in terms of proliferation and invasiveness. Cell cultures co-cultivated or treated with MSCs conditioned media showed inhibited growth [118-120] and co-injection of tumor cells and MSCs in nude animals showed that tumor growth was significantly inhibited [120]. Some authors explain this activity by MSCs to inhibit the expression of Wnt signaling pathway-related factors in tumor cells, consequently unbalancing cellular proliferation and apoptosis [121].

To conclude, the presence of MSC within the tumor site is a fact, but its role is still to be determined.

3. CSCs and therapy outcomes

In pancreatic cancer, surgery is usually accompanied by other complementary treatments such as multi-chemotherapy regimens and radiotherapy. Despite clear progress in detection and treatment of cancer, current strategies fail to completely remove the tumor and prevent recurrence and metastasis. Existing therapies are toxic and non-specific, being directed towards both normal cells and tumor cells. Most chemotherapeutic regimens are based on gemcitabine, but provided a modest improvement in median survival. The response rate was increased by using more than two chemotherapeutic agents [122]. Human pancreatic cancer tissue contains CSCs defined by CD133 and CXCR4 expression and these cells are highly resistant to standard chemotherapy and are involved in metastasis [12]. Features of CSCs have also been confirmed in brain and colon cancers [9]. Therapy failure for other highly malignant tumors has been explained, at least partially, by the chemo-[10, 123] and radio-resistant [124] nature of CSCs. Cancer stem cells therapy resistance is considered to be the result of inappropriate activation of several proliferative signaling pathways, including EGFR, PDGFR (platelet-derived growth factor receptor), stem cell factor (SCF) receptor KIT [125], and activation of Hedgehog and Wnt/ β -catenin signaling [50]. Another well sustained argument for chemotherapy resistance is the expression of multidrug resistance-linked genes, out of which most are ATP-binding cassette (ABC) drug transporters [126]. High levels of ABC transporters were documented in pancreatic CSCs and chemotherapeutic agents such as etoposide, doxorubicin, vincristine and paclitaxel are direct substrates of ABC transporters [127]. Gemcitabine uptake, the golden standard for pancreatic adenocarcinoma chemotherapy, seems to be negatively influenced by expression of ABCG2, though there is no clear evidence that ABC transporters directly efflux gemcitabine or its metabolites in pancreatic cancer cells [90]. Several reports indicate that conventional chemotherapy itself could propagate the CSC population in pancreatic cancer, through exerting a positive selection pressure of CD24/CD44/ESA triple positive CSC fraction [12, 128].

Differential expression of some CSCs biomarkers can be indicative of particular characteristics, such as responsiveness to different therapies or outcomes.

3.1. CSCs as therapeutic targets

Different strategies are developed to target specifically CSCs, thus eliminating this particular set of cells. Several key regulatory pathways operating in the stem cells have been proposed and demonstrated to considerably improve the therapy outcomes; relevant examples are Sonic Hedgehog, Notch/Jagged, CD133, TGF beta signaling; specifically addressing such pathways, by small molecule inhibitors, monoclonal antibodies or siRNAs results in increasing the efficacy of therapies, as suggested by *in vitro* studies, as well as by clinical outcomes.

Some *in vitro* studies showed that blocking *cis*-acting elements, that are common for pluripotency maintaining Transcription Factor SOX-2 (Sox2), Oct4, and proto-Oncogene C-Myc (c-Myc), dramatically decreased CSCs proliferation and their ability to generate tumors in nude mice [15]. Equally, simultaneous knockdown of OCT4 and its target Nanog led to decreased proliferation, migration, invasiveness and tumorigenesis of putative pancreatic cancer stem cells [129]. Inhibition of the Nodal/Activin receptor Alk4/7 in CSCs decreased almost to zero their self-renewal capacity and tumorigenicity, and reversed the resistance of CSCs to gemcitabine. Concordant with previous reports on stroma-tumor interaction, Lonardo *et al.* also found the response to gemcitabine was dependent on the amount of stroma which hindered drug delivery. The addition of a stroma-targeting hedgehog pathway inhibitor (HHI) enhanced delivery of the Nodal/Activin inhibitor and translated into long-term, progression-free survival [130].

The *Hedgehog* signaling pathway is usually targeted in experimental designs as adjuvant to classic chemotherapy. The combined blockade of Shh and mTOR signaling together with gemcitabine is capable of eliminating pancreatic CSCs [131]. Inhibition of Smoothen (Smo), combined with gemcitabine and mTOR inhibitor rapamycin, led to abrogation of cancer stem cells and the authors reported a long-term disease stabilization or regression and subsequent long-term survival [132].

Notch pathway inhibition by selective γ -secretase inhibitors, such as PF-03084014, a selective γ -secretase inhibitor, alone and in combination with gemcitabine, inhibited the cleavage of nuclear Notch 1 intracellular domain and Notch targets Hes-1 and Hey-1 and induced tumor regression in xenograft tumor models. The authors argue that the observed effects are due to PF-03084014 targeting of putative aggressive cancer stem cells [59]. Another potent and selective γ -secretase inhibitor, MRK-003, also led to downregulation of nuclear Notch1 intracellular domain, inhibition of anchorage-independent growth, and reduction of tumor-initiating cells capable of extensive self-renewal. Pretreatment of a pancreatic adenocarcinoma cell line with MRK-003 significantly inhibited the subsequent engraftment in immunocompromised mice and mixed regimen MRK-003 and gemcitabine of engrafted mice reduced tumor cell proliferation, and induced both apoptosis and intratumoral necrosis [133]. However, some of such pathways are common to normal and CSCs, raising the problem of increasing the selectivity towards cancer stem cells.

3.2. Clinical studies

Most clinical studies addressing molecular therapies in pancreatic cancer report usage of monoclonal antibodies, for several simple rationales: i) they are already tested as drugs in other types of pathologies, tumoral or not; ii) they block proliferative oversignaling – a characteristic feature of malignancy; iii) some of them address phenotypic anomalies given by genetic dysregulations, such as EFGR overexpression/ oversignaling. However, these antibodies do not address specifically stem cells, but the larger category of cancer cells. There are some constructs that are, however, effective on the side population of CSCs. A combination of tigatuzumab, a fully humanized death receptor5 (DR5) agonist monoclonal antibody, with gemcitabine proved to be more efficacious in killing both CSCs and adenocarcinoma bulk cells.

The combination therapy produced remarkable reduction in pancreatic CSCs, tumor remissions, and significant improvements in time to tumor progression [134]. Signaling pathways can also be inhibited by small molecule kinase inhibitors that act downstream of the extracellular domain of the receptor. Sunitinib targets multiple receptor tyrosine kinases, including stem cell factor receptor (c-KIT) and it has been shown to have antitumor efficacy in *in vivo*. The combination of gemcitabine with sunitinib could not surpass the effects of the single agent sunitinib [135]. Cabozantinib – a small kinase inhibitor that targets c-Met and VEGFR2-inhibited viability and spheroid formation and induced apoptosis in pancreatic malignant cells with minor effects in non-malignant cells. In primary, CSC-enriched spheroidal cultures cabozantinib downregulated CSC markers SOX2, c-Met and CD133 and induced apoptosis [73]. Most clinical studies, so far, do not seem to report any significant improvement with various regimens employed [136]. Early clinical data for the Shh inhibitor, GDC-0449 (vismodegib), in combination with either gemcitabine or erlotinib, indicate that these regimens are feasible and well tolerated [137]. However, a phase II trial of gemcitabine plus saridegib versus gemcitabine plus placebo in previously untreated patients with metastatic pancreatic cancer was halted early based on a shorter overall survival rate in the gemcitabine plus saridegib arm [106].

A very interesting new trend in advanced, chemotherapy-resistant cancers, aiming for a different approach, tests personalized peptide vaccination (PPV) – a method to generate an immune response against tumor-associated antigens and so far employed for aggressive cancers such as lung cancer [138] and biliary tract cancer [139]. For advanced pancreatic cancer a phase II clinical trial was also conducted in which vaccine antigens were selected and administered based on the pre-existing IgG responses to 31 different pooled peptides [140]. Other vaccines are aimed at increasing the patient's immune response against tumor cells – targeting cancer markers with the aid of specialized antigen-presenting cells such as dendritic cells. Currently, there are several vaccines for human pancreatic cancer in clinical trials including: i) whole-cell vaccines, ii) combined dendritic cells with antigen to present to patient leukocytes iii) peptide and DNA vaccines, iv) Ras peptide vaccine; v) vaccine against common cancer mutations, targetable by CD4/8 T cells; vi) Telomerase peptide vaccine; vii) carcinoembryonic antigen (CEA) and Mucin 1; viii) Survivin-targeted vaccine [141]. Also, it was shown that boosting the immune response by additional treatment with dendritic cells (LANEX-DC®) is highly effective and extends the median survival times up to 8.9 months [142].

Lack of response to all of the above mentioned types of therapies led to an investigation of *non-conventional therapies*. Salinomycin, an anti-protozoa agent that was recently shown to preferentially kill breast CSCs [143], and later investigated in other types of malignancies, was shown to inhibit growth of pancreatic adenocarcinoma CSCs *in vitro*. *In vivo* xenografting studies showed that salinomycin combined with gemcitabine could eliminate the engraftment of human pancreatic cancer more effectively than the individual agents [144]. Adamantyl-substituted retinoid-related molecules (ARRs) inhibit growth and induce apoptosis in the pancreatic stem-like cell population, possibly through decreased IGF-1R and β -catenin expression [145]. Isothiocyanate sulforaphane (SF) was used as sensitizer of pancreatic CSCs to tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-induced apoptosis, by quercetin and sorafenib. The combination of SF with a cytotoxic drug efficiently induced

apoptosis along with inhibition of self-renewing potential, ALDH1 activity, clonogenicity, xenograft growth and relapse of gemcitabine-treated tumor cells in nude mice [146]. The flavonoid Quercetin enhances TRAIL-mediated apoptosis, acts as a chemosensitizer for the ABC pump-proteins, and can enhance the effects of sulforaphane in inhibiting the pancreatic CSC characteristics [147].

4. Nanotheragnostics in pancreatic cancer

Targeted therapeutic delivery is a way to ensure that drugs reach the designated target at the highest concentration within safety margins, limiting in the same time undesired side effects resulting from unspecific diffusion in well vascularized tissues. This aim is now being resolved with the use of nanomedicine –a multidisciplinary field that aims to utilize nanoscale (up to 100 nm) particles to improve delivery of chemotherapeutics [148]. These constructs fall into several categories – micelles, microemulsions, liposomes, polymers [149] silica and carbon-based nanoparticles [150] and dendrimers [151]. This coating of a nanoparticle can be improved with stabilizing agents (such as polyethylene glycol – PEG) or ligands to direct them to a specific target (such as an antibody towards a cancer cell type). Liposome delivery of active agents has been recently paired with ultrasound technology, by development of ultrasound-responsive stable liposomes. Ultrasound-induced heating triggers phase transition in the phospholipid membrane, leading to drug release in the targeted region [152]. To date, there are at least twelve FDA (Food and Drug Administration) approved liposome-based drugs, most of them being chemotherapeutics for breast, ovarian and pancreatic cancer [153].

Generation of magnetic/metallic nanoparticles was considered a step-forward in magnetic resonance imaging and diagnostics [154], adding a new utility to biomedical nanoscience. Another type of imaging strategy using nanoparticles is optical, through use of carbon nanomaterials that display natural fluorescence emission [155], or use of other infrared light emission agents [156], forming upconversion nanoparticles [157], or incorporated in a wide variety of coating surfaces, such as gold [158] and polymer-based [159]. Photoacoustic imaging is another nanomedical promising technology that combines the benefits of optical imaging methods with the clinically available and cost-effective ultrasound imaging modality [160]. Originally used for investigation of vascularization pattern, based on high endogenous contrast of blood *versus* surrounding tissues [161] and or/vascular wall/lumen alterations [162], it has been increasingly used in tumor assessment, providing further molecular information on cancer, given by the chemical composition of tissues and by targeted nanoparticles that can interact with extravascular tissues at the receptor level [163].

By incorporating active drugs into imaging nanoparticles, a dual therapeutic and diagnostic agent was generated, thus the emerging field of "theragnostic", is widely used especially in cancer research. Most nanoparticles accumulate in tumors due to their intense and leaky neovascularization, but some can be retained there with the use of cancer-specific antigens [164] and stimulated into releasing their chemotherapeutic cargo. Cancer diagnostic and concomitant treatment through nanoparticles benefits from real-time assessment of drug bioavailability and more accurate monitoring of tumor evolution.

Pancreatic cancer treatment benefits from development of biomedical nanotechnology, in both clinical practice and fundamental research. A PEGylated polymeric nanoparticle containing a potent antagonist of the Hedgehog transcription factor Gli1 combined with gemcitabine significantly impeded the growth of orthotopic pancreatic cancer xenografts [165]. In *in vivo* studies, squalene-conjugated gemcitabine nanoparticles decreased tumor growth significantly, prevented tumor cell invasion, and prolonged the survival time of mice bearing orthotopic pancreatic tumors [166]. Liposomal delivery of tissue transglutaminase 2 siRNA effectively blocked the growth of pancreatic adenocarcinoma in nude mice [167]. EGFR monoclonal antibody or peptidylglycine alpha-amidating monooxygenase (PAM4)-conjugated gold nanoparticles induced significant tumor destruction in a murine model of pancreatic carcinoma after radiofrequency radiation [168]. Paclitaxel, one of first-line chemotherapeutic agents before the gemcitabine era, is now available as a positively charged lipid-based complex (known as EndoTAG-1) [169] that in combination with gemcitabine was able to inhibit the incidence of metastasis in pancreatic cancer animal models [170]. A controlled phase II clinical trial for pancreatic cancer showed significantly increased survival rates of patients treated with EndoTAG®-1 and gemcitabine combination therapy [171]. An ongoing phase I study (NCT00968604) of advanced pancreatic cancer is currently investigating the effects of intravenous injection of the liposome nanoparticle BikDD, which contains a pro-apoptotic agent [172].

4.1. Nanoparticles for cancer stem cell targeted therapy

In the same manner that nanoparticles are targeted for the bulk tumor, they can be targeted for CSCs, through the use of antigens against specific CSCs markers (e.g CD-133). Such targeted therapy has already been tested *in vitro*, against targeting CD133-expressing cancer cells of colon and pancreatic origin, with encouraging results [56]. Breast CSCs-targeted nanoparticle delivery of doxorubicin reduced their mammosphere formation capacity and cancer initiation activity, eliciting tumor growth inhibition in animal models [173].

Apart from cytotoxic drug delivery, nanoparticles can be used to target and modify certain characteristics of CSCs, such as activation of signaling pathways that confer renewal properties, targeting metabolism and inhibiting drug efflux transporters in an attempt to sensitize them to therapy [174]. Multi-lamellar vesicle liposomes targeted against CSCs, containing a steroid nucleus, were formulated to disrupt mitochondrial integrity and to facilitate release of cytochrome c to attain programmed cell death [175].

5. Conclusions

CSCs represent key components in the heterogeneous cellular system represented by pancreatic tumors. Their biological features configure them as one of the major players and major targets for investigation; they offer sets of additional and reliable biomarkers for prognosis and stratification. Discovery of target mechanisms and molecules within cancer stem cells is plausible to provide the needed boost for therapy improvement.

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